Bleb Formation in Hepatocytes During Drug Metabolism Is Caused by Disturbances in Thiol and Calcium Ion Homeostasis

Abstract. *A wide variety of toxic chemicals cause blebbing of the plasma membrane in isolated hepatocytes. These alterations in surface structure occur well before cell death. The formation of blebs appears to be directly related to changes in the concentration of extramitochondrial calcium ions. These changes probably reduce the ability of the hepatocyte cytoskeleton to maintain normal surface morphology. The concentration of soluble thiols, notably glutathione, appears to regulate the size of the extramitochondrial calcium ion pool. Disturbances in intracellular thiol and calcium ion homeostasis therefore seem to be responsible for the surface blebbing observed during toxic injury to isolated hepatocytes.*

One of the earliest responses of isolated cells to chemical and ischemic injury is the formation of blebs in the plasma membrane (*1*). For example, we have shown that plasma membrane blebbing is an early sign of bromobenzene toxicity in isolated rat hepatocytes (*2*). The depletion of reduced glutathione (GSH) by bromobenzene appears to elicit this change in the hepatocyte surface structure (*2*). The formation of such blebs probably reflects a change in cytoskeletal structure, because substances that interact directly with the hepatocyte cytoskeleton, such as cytochalasins B and D and phalloidin, produce blebs of similar appearance (*3*). Drugs that do not interact directly with the cytoskeleton could cause bleb formation by altering intracellular Ca^{2+} homeostasis, since Ca^{2+} and its associated binding proteins play a pivotal role in regulating cytoskeletal structure and function (*4*). The structure of the hepatocyte cytoskeleton could be affected by a change in the cytosolic Ca^{2+} concentration, like that produced by an increased influx of Ca^{2+} through a damaged, and therefore more permeable, plasma membrane. We recently showed, however, that three differently acting liver cell toxins, including bromobenzene, are more toxic to isolated hepatocytes in the absence of extracellular Ca^{2+} than in its presence (*5*). We also observed that the absence of extracellular Ca^{2+} greatly enhances surface blebbing in the presence of toxic substrates. This suggests that a change in the intracellular distribution of Ca^{2+} is responsible for the observed alterations in surface morphology and that an influx of extracellular Ca^{2+} is not involved. We report here that an influx of extracellular Ca^{2+} is not responsible for the surface blebbing. Changes in extramitochondrial Ca^{2+} are the causative factor.

We recently developed a method for measuring Ca^{2+} in different compartments of isolated hepatocytes (*6*). The method, which is based on techniques described by Murphy *et al.* (*7*), involves the use of the uncoupler carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), the ionophore A23187, and the metallochromic indicator arsenazo III (*6*). The Ca^{2+} released by FCCP represents the mitochondrial pool of Ca^{2+} and the Ca^{2+} released by A23187 (after FCCP-induced Ca^{2+} release) represents the extramitochondrial pool (*6*). We incubated rat hepatocytes with certain drug substrates in buffered balanced salt solution containing 2.6 m*M* Ca^{2+} and at specific times determined the mitochondrial and extramitochondrial Ca^{2+} concentrations. In control hepatocytes approximately 58 percent (2.1 ± 0.2 nmole of Ca^{2+} per 10^6 cells) of the total releasable Ca^{2+} was located in the mitochondrial compartment and 42 percent (1.5 ± 0.2 nmole per 10^6 cells) was located extramitochondrially. These results are in close agreement with those of other investigators (*7, 8*), who determined the distribution of Ca^{2+} in hepatocytes by a variety of techniques.

Incubation of hepatocytes with bromobenzene (0.6 m*M*) resulted in a progressive loss of intracellular GSH (Fig. 1). This had no effect on the mitochondrial Ca^{2+} pool but caused a concomitant loss of extramitochondrial Ca^{2+}. The depletion of GSH and extramitochondrial Ca^{2+} brought about a slow but progressive increase in surface blebbing (Table 1). The uncoupler dicoumarol (bishydroxycoumarin) had only a small effect on intracellular GSH (Fig. 1). At a final concentration of 30 μ*M* it rapidly depleted the mitochondrial Ca^{2+} pool but caused only a small (yet significant) depletion of extramitochondrial Ca^{2+}. However, it did not cause surface blebbing at this concentration (Table 1), suggesting that (i) the mitochondrial Ca^{2+} concentration bears no direct relation to the surface morphology of hepatocytes and that (ii) the concentration of extramitochondrial Ca^{2+} must fall below a certain threshold before blebbing occurs. Dicoumarol also inhibits reduced nicotinamide adenine dinucleotide phosphate: (quinone-acceptor) oxidoreductase (E.C.1.6.99.2) in the liver, prevent-

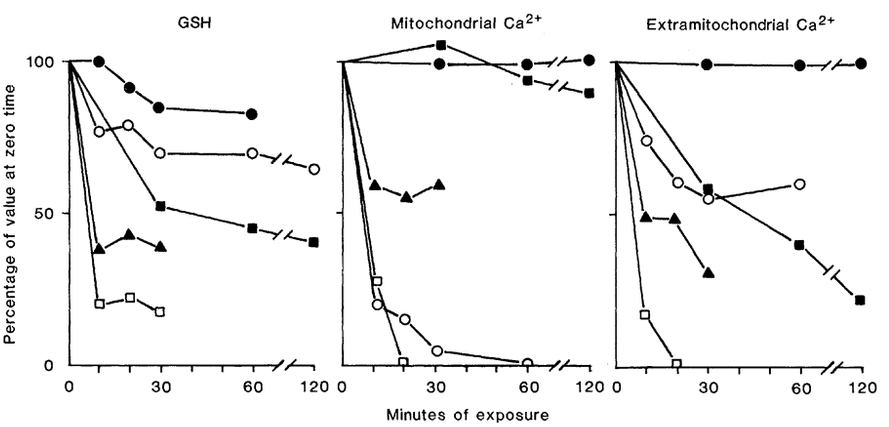

Fig. 1. Modification of Ca^{2+} concentrations in isolated hepatocytes by substrates causing GSH depletion during their metabolism. Hepatocytes were isolated from male Sprague-Dawley rats (180 to 200 g) by collagenase perfusion (*14*). The rats had received sodium phenobarbital (1 mg/ml) in their drinking water for 1 week prior to the isolation of hepatocytes. The hepatocytes were incubated (*15*) at a concentration of 6 × 10^6 cells per milliliter in Krebs-Henseleit buffer (*p*H 7.4) supplemented with 12.6 m*M* Hepes. After 15 minutes zero-time samples were taken and the substrate was added. GSH levels were determined by a colorimetric method (*16*) or by high-pressure liquid chromatography (*17*). For the measurement of intracellular Ca^{2+}, cells were separated from the Ca^{2+}-containing incubation medium by rapid centrifugation through a suspension of Percoll in Ca^{2+}- and Mg^{2+}-free Hanks medium (final density, 1.06 g/ml) (*6, 7*). The hepatocytes were quickly resuspended in the modified Hanks medium and two samples were taken: one for cell enumeration and the other for Ca^{2+} measurement. The amount of Ca^{2+} in each of the intracellular pools was determined in 4 × 10^6 cells as follows. First, 5 μ*M* FCCP was added to release the Ca^{2+} from the mitochondrial pool. After stabilization of the Ca^{2+} level 7.5 μ*M* A23187 was added to release the extramitochondrial Ca^{2+} into the medium. Zero-time values were 50.6 ± 3.6, 2.1 ± 0.2, and 1.5 ± 0.2 nmole per 10^6 cells for GSH, Ca^{2+} in the mitochondrial pool, and Ca^{2+} in the extramitochondrial pool, respectively. Symbols: (●) control, (■) 0.6 m*M* bromobenzene, (▲) 4 m*M* *t*-butyl hydroperoxide, (○) 30 μ*M* dicoumarol, and (□) 30 μ*M* dicoumarol plus 50 μ*M* menadione.

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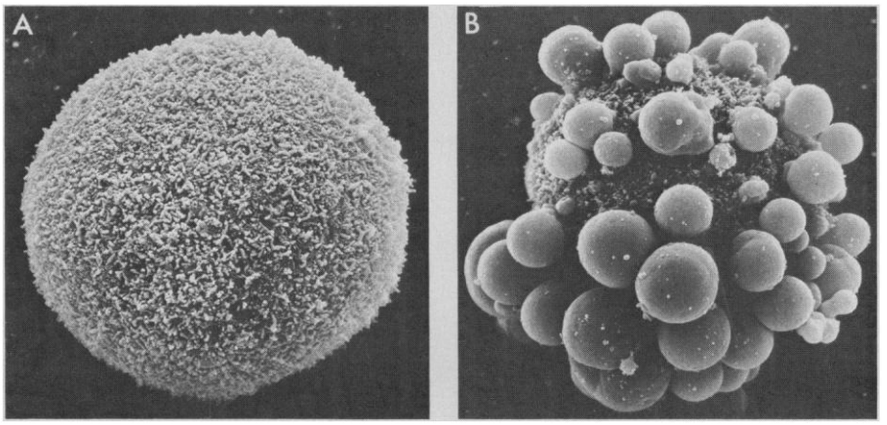

Fig. 2. Scanning electron micrographs of typical hepatocytes incubated for 30 minutes in Krebs-Henseleit buffer (*p*H 7.4) supplemented with 12.6 m*M* Hepes in the absence (A) or presence (B) of 30 μ*M* dicoumarol plus 50 μ*M* menadione. The samples were processed in accordance with standard procedures involving glutaraldehyde and osmium fixation followed by critical point drying and visualization in a JEOL scanning electron microscope (model JSM 35). Magnifications: (A) x4000 and (B) x2500.

ing the two-electron reduction of certain quinones and making more quinone available for single-electron reduction, which can produce active oxygen species (*9*). The incubation of hepatocytes with dicoumarol and menadione (2-methyl-1,4-naphthoquinone) leads to a rapid depletion of GSH and both intracellular Ca^{2+} pools (Fig. 1). There is also a massive increase in the incidence of surface blebbing (Fig. 2 and Table 1), providing support for suggestion (ii) above and indicating that changes in both Ca^{2+} pools are required for rapid and extensive blebbing to occur.

t-Butyl hydroperoxide alters the redox state of pyridine nucleotides in mitochondria (*6, 10*) and depletes the mitochondrial Ca^{2+} pool in hepatocytes (Fig. 1). It also acts as a substrate for cytosolic glutathione peroxidases, causing the oxidation of GSH and the release of oxidized glutathione into the extracellular medium (*11*). This rapid oxidation of intracellular GSH brings about the loss of extramitochondrial Ca^{2+} and the appearance of cell surface blebs (Table 1).

The ionophore A23187 promotes the movement of Ca^{2+} across cellular membranes and down concentration gradients. In the presence of millimolar concentrations of extracellular Ca^{2+} it therefore causes the rapid entry of Ca^{2+} into cells. This did not, however, cause the appearance of blebs on the surface of the isolated rat hepatocytes (Table 1), nor did it alter their viability. This lends further support to our conclusion that toxic injury to isolated hepatocytes is not dependent on the influx of extracellular Ca^{2+} (*7*) and suggests that interference with intracellular Ca^{2+} homeostasis is more important for toxicity. Indeed, the incubation of hepatocytes with A23187 in the absence of extracellular Ca^{2+} caused a rapid loss of both mitochondrial and extramitochondrial Ca^{2+}, the appearance of blebs at the cell surface (Table 1), and loss of viability.

We conclude that the appearance of blebs in the plasma membrane of isolated hepatocytes during toxic injury is related to the loss of extramitochondrial Ca^{2+}. Moreover, the concentration of extramitochondrial Ca^{2+} appears to be controlled by the thiol status of the cells and possibly also by the mitochondria. Further support for the idea that thiols are protective is given by the fact that dithiothreitol, a well-known thiol reagent, completely prevents both extramitochondrial Ca^{2+} loss and surface blebbing during *t*-butyl hydroperoxide metabolism in hepatocytes (*6*). Soluble thiols, notably GSH, may protect by (i) preventing the inactivation of the Ca^{2+}-binding proteins, such as calmodulin, which has functionally important methionine groups (*12*) and (ii) by maintaining the thiol groups of intracellular membrane proteins in their normal state. Recent results from our laboratory suggest that the latter is especially important for the maintenance of the Ca^{2+}-sequestering function of the endoplasmic reticulum (*13*). Inactivation of Ca^{2+}-binding proteins and destruction of Ca^{2+} sequestration in the endoplasmic reticulum would disturb intracellular Ca^{2+} homeostasis and inhibit enzymes associated with the cytoskeleton, such as actomyosin adenosine triphosphatase.

Table 1. Onset of plasma membrane blebbing after treatment with substrates affecting the concentrations of cellular GSH and Ca^{2+}. Hepatocytes were isolated and incubated with the substrates as described in the legend to Fig. 1. When A23187 was used the hepatocytes were incubated in Krebs-Henseleit buffer or washed and incubated in Ca^{2+}- and Mg^{2+}-free Hanks solution (*7*). At the times indicated cell samples were viewed under a light microscope. Symbols: (−) percentage of cells affected by blebbing not significantly greater than that in control incubations, (+) blebs affect 30 percent of the cells, (++) blebs affect 31 to 70 percent of the cells, and (+++) blebs affect over 70 percent of the cells.

Treatment	Exposure period (minutes)			
	0	30	60	120
Dicoumarol (30 μ*M*)	−	−	−	−
A23187 (30 μ*M*)	−	−	−	−
Bromobenzene (0.6 m*M*)	−	−	+	++
Dicoumarol (30 μ*M*) plus menadione (50 μ*M*)	−	+++		
t-Butyl hydroperoxide (4 m*M*)	−	+++		
A23187 (30 μ*M*) in Ca^{2+}-free medium	−	+++		

In conclusion, early changes in thiol and Ca^{2+} homeostasis cause the surface blebbing observed during toxic injury to isolated hepatocytes. These may be important primary events in the action of toxins on liver cells.

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18. Supported by grants from the Swedish Medical Research Council (project 03X-2471), the Swedish Council for Planning and Coordination of Research, and the Nobel foundation. We thank S. Thorold for preparing the scanning electron micrographs and P. Hartzell for expert technical assistance.

4 March 1982; revised 4 June 1982

Polyamines and Plant Stress: Activation of Putrescine Biosynthesis by Osmotic Shock

Abstract. *The putrescine content of oat leaf cells and protoplasts increases up to 60-fold within 6 hours of exposure to osmotic stress (0.4 to 0.6 molar sorbitol). Barley, corn, wheat, and wild oat leaves show a similar response. Increased arginine decarboxylase activity parallels the rise in putrescine, whereas ornithine decarboxylase remains unchanged.* DL-α*-Difluoromethylarginine, a specific irreversible inhibitor of arginine decarboxylase, prevents the stress-induced rise in increase in arginine decarboxylase activity and putrescine synthesis, indicating the preferential activation of this pathway.*

Although leaf mesophyll protoplasts of many species have been successfully cultivated and regenerated in vitro, cereal protoplasts have remained refractory (*1*). The addition of the polyamines putrescine, spermidine, and spermine to the isolation and culture medium stabilizes oat leaf protoplasts against lysis, increases their incorporation of [^{3}H]leucine and [^{3}H]uridine into macromolecules (*2*), induces limited mitosis (*3*), and retards leaf senescence (*4*). These observations prompted us to study the role of polyamines in the physiology of plants.

The pattern of polyamines in freshly isolated oat mesophyll protoplasts is strikingly different from that in mesophyll cells. The titer of putrescine, as confirmed by high-performance liquid chromatography and thin-layer chromatography (*5*), increases five- to tenfold in protoplasts, whereas spermidine and spermine remain constant or show a slight decrease. Since the osmoticum present in the protoplast isolation medium (*1*) is known to trigger an increase in ribonuclease and protease activities (*2*), changes characteristic of leaf senescence (*4*), we tested the possibility that the increase in putrescine titer is a direct result of the osmotic treatment.

The lower epidermis of primary leaves of 7- to 9-day-old oat cv. Victory seedlings was removed and the leaves floated under light in the presence and absence of 0.6*M* sorbitol, an osmoticum commonly used during protoplast isolation (*1, 3*). A significant increase in putrescine was evident after 2 hours of such incubation, and a level 60-fold higher than the initial value was reached by 6 hours (Fig. 1A). In contrast, putrescine concentrations were not significantly altered by incubation of leaf segments in phosphate buffer (1 m*M*, *p*H 5.8). It is unlikely that the osmoticum-induced increase in putrescine is due to its release from a bound form, since acid hydrolysis of the perchloric acid–soluble and insoluble fractions of leaf extracts causes little or no putrescine release after 16 hours at 110°C in 6*N* HCl (*6*). The other polyamines, spermidine and spermine, show a gradual decline during osmotic treatment, with a concomitant increase in 1,3-diaminopropane, the product of their oxidation by the polyamine oxidase present in cereal leaves (*7*). Osmotica such as mannitol, proline, betaine, and to a lesser extent sucrose, have a similar effect on the putrescine titer.

The response of putrescine to osmotic shock is not restricted to oat. Leaf segments of wild oat, barley, corn, and wheat floated on 0.4*M* sorbitol also showed significant increases in putrescine after 4 hours (Fig. 1B). The putrescine concentration at the start of the experiment was not significantly different from that of the control leaves incubated in buffer for 4 hours; however, there is a twofold increase in putrescine in corn leaf segments floated on buffer, possibly because of the damage induced by peeling. Wheat leaf segments, which cannot be peeled and do not show any obvious signs of wilting in the presence of osmoticum, show a 4.6-fold increase in putrescine titer (Fig. 1B). Thus, injury due to peeling is not essential for the increase in putrescine. Also, the threshold levels of osmoticum necessary for the response are probably much lower than the concentrations used in these experiments, since treatment with even 0.1*M* sorbitol increased the putrescine titer 2.5-fold after 4 hours. Increased putrescine levels have also been noted during deficiencies of potassium (*8, 9*) and magnesium (*9*), acid stress, (*6, 10*) and NH_4Cl feeding (*11*). Bacterial and mammalian cells, however, show an inverse correlation between polyamine content (putrescine, spermidine, and spermine) and the osmolality of the culture medium (*12*).

In contrast with mammalian cells, in

Table 1. Polyamine titer and biosynthetic enzyme activity in oat leaf segments under osmotic stress. Peeled leaf segments (7 days old) were floated in buffer (control) or buffer plus 0.6*M* sorbitol in the presence or absence of polyamine inhibitors. After 4 hours of incubation at 25 to 26°C in darkness, triplicate samples were taken for polyamine analysis (*5*) and enzyme assay. Enzymes were extracted at 5°C in 0.1*M* phosphate buffer, *p*H 7.8. The clear supernatant obtained after centrifugation at 26,000*g* for 20 minutes was used. Assay conditions are described in (*14*). The results for the polyamine concentrations are expressed as means ± standard error. Numbers in parentheses express enzyme activities as percentages of the 4-hour control values. Protein was determined according to the Coomassie blue G-250 assay (*23*), with gamma globulin being used as the standard.

Treatment	Polyamine (nmole/g fresh weight)			Enzyme activity per hour (nmole of CO_2/mg protein)	
	Putrescine	Spermidine	Spermine	Arginine decarboxylase	Ornithine decarboxylase
Control (at 0 hour)	16 ± 1	161 ± 18	41 ± 4	2.27 (115)	4.24 (93)
Control (at 4 hours)	13 ± 2	152 ± 10	38 ± 3	1.98 (100)	4.55 (100)
Sorbitol	102 ± 5*	120 ± 13†	21 ± 2*	3.81 (192)*	4.65 (102)
Sorbitol plus $10^{-3}M$ DFMO	129 ± 7*	186 ± 10†	32 ± 3	5.31 (268)*	4.68 (103)
Sorbitol plus $10^{-4}M$ DFMA	20 ± 2†	123 ± 7†	36 ± 2	1.24 (63)†	4.64 (102)
Sorbitol plus $10^{-3}M$ DFMA	10 ± 1	128 ± 8	32 ± 2	0.41 (21)*	4.55 (100)

*Significantly different from control at 4 hours ($P < .01$). †Significantly different at $P < .05$.

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